Gut, 1982, **23**, 173–180

Leucocyte function in Crohn's disease*

Studies on mobilisation using a quantitative skin window technique and on the function of circulating polymorphonuclear leucocytes *in vitro*

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SUMMARY Leucocyte function was evaluated by mobilisation to skin windows with chambers and by the chemotactic, phagocytic, and nitro blue tetrazolium (NBT) reducing activity of circulating leucocytes *in vitro* in 20 patients with Crohn's disease, 21 healthy volunteers, and nine patients with sarcoidosis or tuberculosis. Leucocytes had been mobilised in significantly reduced numbers at 12, 24, 36, and 48 hours in Crohn's disease compared with healthy volunteers ($P<0.01$) and patients with sarcoidosis/tuberculosis ($P<0.01$). The leucocyte migration rate showed that mobilisation in Crohn's disease begins after a prolonged lag phase and is reduced compared with healthy volunteers ($P<0.01$) and patients with sarcoidosis/tuberculosis ($P<0.02$). The reduced mobilisation was not correlated with disease activity. *In vitro* random migration by leucocytes was slightly lower in Crohn's disease ($P<0.05$) than in healthy volunteers, but there was no difference after removal of the autologous plasma. Chemotactic response to casein did not differ between the groups studied. Serum independent and dependent phagocytosis did not differ from control groups. Serum independent phagocytosis was positively and significantly correlated to the disease activity (rho 0.4812, $P<0.05$). Resting leucocyte NBT reduction was increased in Crohn's disease and sarcoidosis/tuberculosis ($P<0.01$), but during phagocytosis a lower NBT reduction was found in Crohn's disease than in healthy volunteers ($P<0.02$). The inflammatory response in Crohn's disease, with reduced leucocyte accumulation, differs from patients with other granulomatous reactions and is independent of the disease activity. Our data suggest that the defect is not cellular. They support the hypothesis that a pathogenic factor in Crohn's disease may be foreign material that is normally eliminated remaining in the tissue and eliciting a chronic inflammatory response.

Epitheloid cell granulomas with Langerhans's type giant cells are—though not obligatory—a predominant and distinguishing feature of the inflammatory reaction in Crohn's disease.

A similar histological appearance is found in infection with mycobacteria and fungi or in the foreign body reaction. The granulomatous reaction is assumed to be the result of persistent challenge by foreign material within the phagocytes.[1]

A condition with abnormal phagocyte function—for example, chronic granulomatous disease—shows similar histology with granulomas in the reticuloendothelial system. In chronic granulomatous disease a defective oxygen dependent bactericidal function allows bacteria and fungi to remain viable within the polymorphonuclear neutrophilic granulocytes and macrophages. In some cases the granulomatous reaction has been confined to the gastrointestinal tract.[2]

A defective primary defence system in Crohn's disease is an open possibility on this background.

The introduction of a quantitative skin window technique[3] facilitated studies of the leucocyte mobilisation *in vivo*. Segal and Loewi[4] applied this technique in patients with Crohn's disease and found a reduced leucocyte mobilisation. Gange *et al.*[5] found reduced leucocyte mobilisation in sarcoidosis also.

The aim of the present study was to investigate the total number of leucocytes mobilised to an experimental inflammatory lesion as well as the kinetics of the mobilisation. It included also determinations of the function of the circulating

*Presented in part at the World Congress of Gastroenterology, June 1978, Madrid, Spain.

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Received for publication 28 July 1981

leucocytes—namely, their chemotactic, phagocytic, and peroxidative metabolic activity.

Methods

PATIENTS

Twenty patients with Crohn's disease were studied (11 females, nine males), median age 32 years (range 13–74 years). No patient was on medication at the time of study. Any previous treatment with steroids had been discontinued for more than three months, sulphasalazine for two weeks, and all other drugs a minimum of one week before the experiment.

The diagnosis had been established by previously described criteria.[6] Fifteen patients had histologically verified disease, while five had a typical clinical history and typical radiological or endoscopic findings.

The disease involved the small bowel only in five patients, the large bowel in five, and both small and large bowel in 10.

Disease activity was assessed by a clinical activity index.[7]

A score of less than five was found in 13 patients, indicating inactive or only slightly active disease. Five patients had a score of 5–10 and only two of more than 10. The duration of the disease had been 0–25 years (median eight years). Six patients had undergone resection of the intestine more than six months before the study.

CONTROLS

Twenty-one healthy volunteers (10 females, 11 males)—median age 30 years (range 20–68 years)—served as controls. In addition, nine patients with sarcoidosis or tuberculosis made up a group of patients with a granulomatous inflammatory process of different aetiology and location. The diagnosis of sarcoidosis had been confirmed by mediastinoscopy with lymph node biopsy. Four patients had active tuberculosis affecting the chest (two patients), kidney (one patient), and the knee joint (one patient). All controls were off medication, non-febrile, and had normal serum creatinine. If steroids had been given previously, they had been discontinued for at least six months.

All patients gave their informed consent to taking part in the study.

PRACTICAL PROCEDURES

All patients and controls were admitted to hospital for 48 hours. Three hours after admission a skin window was prepared. Venous blood was obtained immediately after admission and 24 hours later for measuring the *in vitro* function of polymorphonuclear neutrophilic granulocytes. A total of 150 ml blood was drawn during 48 hours. Results given here are the values found at 24 hours, as the patients were then in the fasting state and had been acclimatised to the hospital environment.

TECHNIQUES

In vivo mobilisation of leucocytes

An epidermal abrasion (2·2–4·8 cm^2; median 3·6 cm^2) was made with a surgical scalpel until the papillary layer was visible as vividly red pin points and the area was uniformly glossy. No bleeding was allowed. The abrasion was covered with an epoxy chamber filled with autologous serum. The chamber medium was drained at fixed intervals for 48 hours and refilled with unused serum as previously described.[8] The leucocytes from the chamber medium were obtained by centrifugation, counted in haemocytometers, and a differential count was performed on May-Grünwald-Giemsa stained coverslip preparations.

The mobilisation is expressed as the cumulated leucocyte migration—namely, the total number of leucocytes per cm^2 abraded area after 12, 24, 36, and 48 hours. The kinetics of the mobilisation is expressed as the leucocyte migration rate—namely, the number of leucocytes mobilised to the chamber per hour per cm^2 abraded area.

In vitro leucocyte function

The function of the granulocytes *in vitro* was studied by previously described methods.[8a]

Chemotactic assay Leucocytes isolated from heparinised venous blood were layered on cellulose ester filters (3 μm, Millipore) and incubated in modified Boyden chambers (three hours, 37°C). The chemotactic response of leucocytes in autologous plasma and of leucocytes in Hanks's balanced salt solution pH 7·3 (HBSS) was measured. Casein (5 mg/ml) was chosen as chemoattractant, as its effect is independent of complement and serum factors. Random migration of leucocytes was measured in chambers containing only Hanks's solution.

The migration was quantified by the leading front method, giving the maximal distance migrated (μm) by at least two leucocytes in each of 10 microscopic fields (400×, Leitz Wetzlar Orthoplan), and by the chemotactic index giving the number of leucocytes located 50 μm inside the filter per thousand leucocytes on the starting surface.

Phagocytosis Leucocytes isolated from blood drawn with acid-citrate-dextrose were incubated for four minutes with paraffin oil emulsion stained

with Oil Red O. Uningested oil was removed by centrifugation, the phagocytosed oil extracted into p-dioxane and measured colorimetrically. The amount of oil phagocytosed was calculated and the results expressed and the amount of oil phagocytosed in mg per minute per 10^7 phagocytes. Paraffin oil emulsion prepared with bovine serum albumin was used to measure the phagocytosis independent of serum factors, while emulsion prepared with lipopolysaccharide from *E. coli* O26B6 and opsonised with fresh autologous serum showed the serum dependent phagocytosis.

Reduction of nitro blue tetrazolium As an indicator of the peroxidative metabolism the generation of formazan from tetrazolium was measured. Nitro blue tetrazolium reduction was determined for resting leucocytes and for leucocytes phagocytosing opsonised lipopolysaccharide emulsion. Leucocytes were incubated with the tetrazolium solution, and the formazan generated was extracted from the cells into p-dioxane and measured colorimetrically. Resting leucocyte activity is expressed as the change in extinction value per 8 min incubation per 10^7 phagocytes. Nitro blue tetrazolium reduction by leucocytes during phagocytosis was corrected for differences in phagocytosis and for resting leucocyte activity and expressed as the change in extinction value per mg phagocytosed oil.

Results

Table 1 shows the concentration of leucocyte subpopulations in patients with Crohn's disease and in the controls. No differences were found between the groups studied.

LEUCOCYTE MOBILISATION

Table 2 summarises the results of the cumulated leucocyte migration. In patients with Crohn's disease a significantly reduced number of leucocytes had been mobilised after 12, 24, 36, and 48 hours compared with the healthy volunters ($P<0.01$), and also to patients with sarcoidosis/tuberculosis ($P<0.01$). The cumulative leucocyte migration values of Crohn's disease patients were unrelated to the clinical activity score (Fig. 1) as well as to the duration and location of the disease.

Patients with sarcoidosis/tuberculosis also exhibited a reduced cumulative leucocyte migration compared with the healthy volunteers.

The leucocyte migration rate provides a measure of the kinetics during leucocyte mobilisation, depicted in Figs. 2 and 3 for patients and controls.

Patients with Crohn's disease mobilise leucocytes to the chambers after a prolonged lag phase, followed by leucocyte migration values consistently lower than in healthy controls ($P<0.01$) and in patients with sarcoidosis/tuberculosis ($P<0.02$).

The kinetics of the mobilisation in patients with Crohn's disease were different from patients with sarcoidosis/tuberculosis. In sarcoidosis and in tuberculosis the mobilisation did not differ from that in healthy controls during the first 22 hours, while mobilisation was depressed during the subsequent 24 hours.

CELLS IN CHAMBER MEDIUM

The leucocytes in the chamber were predominantly polymorphonuclear neutrophilic granulocytes with a higher mean nuclear segmentation than those in the peripheral blood (Table 3). The significantly raised percentage of macrophages in Crohn's disease merely reflects a normal absolute number

Table 1 *Concentration of leucocyte subpopulations*

	N	*Polymorphonuclear neutrophils*		*Lymphocytes*	*Monocytes*	*Eosinophils*
		10^9/l	*Nuclear segmt.*	*10^9/l*	*10^6/l*	*10^6/l*
Crohn's disease	20	3·44 (1·89–11·52)	2·47 (1·7–3·31)	2·28 (1·53–4·16)	192 (0–856)	116 (0–539)
Sarcoidosis/TB	9	2·70 (1·58–5·31)	2·65 (1·83–3·76)	1·87 (0·73–2·95)	234 (43–674)	152 (34–314)
Healthy volunteer	21	2·95 (1·42–4·49)	2·50 (2·05–3·43)	2·04 (1·20–3·85)	194 (0–682)	85 (0–284)

Median value and observed range. No significant differences were found between the groups (Mann-Whitney rank sum test for unpaired data).

Table 2 *Cumulated leucotyte migration during 48 hours after preparing skin window*

	Hours			
	12	*24*	*36*	*48*
Crohn's disease	11 (2–30)*	35 (7–54)*	62 (13–97)*	97 (20–171)*
Sarcoidosis/TB	30 (13–36)	75 (35–111)	114 (58–255)*	145 (78–355)*
Healthy volunteers	20 (10–34)	74 (50–113)	145 (102–261)	200 (160–413)

Median value and observed range in 10^6 leucocytes per cm^2. Values reduced compared with healthy volunteers are indicated by *$P<0.01$ (Mann-Whitney rank sum test for unpaired data).

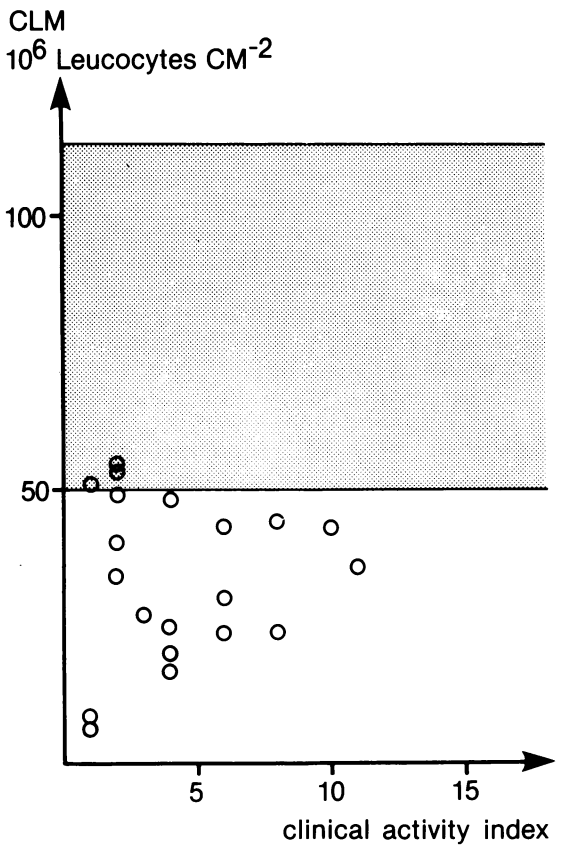

Fig. 1 *Cumulated leucocyte migration (CLM) after 24 hours and clinical activity index in patients with Crohn's disease. The hatched area indicates the range of cumulated leucocyte migration in healthy volunteers.*

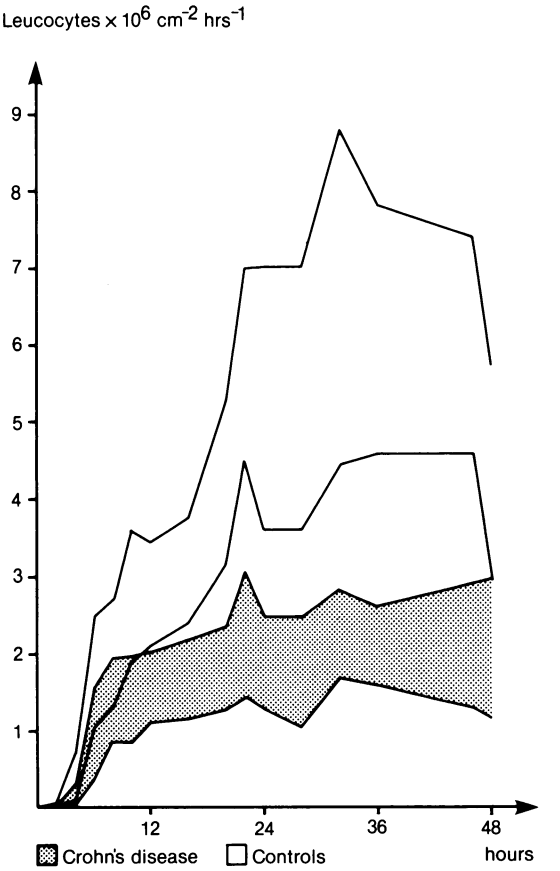

Fig. 2 *Leucocyte migration rates in patients with Crohn's disease and in healthy volunteers. The area indicated gives the 25/75 percentile.*

(Crohn's disease: median 11×10^4/h/cm^2 range 2–27×10^4; healthy volunteers: median 12×10^4/h/cm^2 range 2–49×10^4) not differing from that in patients with sarcoidosis/tuberculosis (median 11×10^4/h/cm^2 range 2–40×10^4). After 28 hours no differences were found between the groups.

IN VITRO FUNCTION OF LEUCOCYTES

Chemotactic migration

Random migration of leucocytes is given in Fig. 4. Measured by the leading front method patients with Crohn's disease showed a random migration of 73 μm (median value) which is slightly lower than

Table 3 *Cellular components of chamber medium at 10–12 and 24–28 hours after start of skin window*

	Polymorphonuclear neutrophils		*Lymphocytes*	*Macrophages*
	Percentage	*Nuclear segmt.*	*Percentage*	*Percentage*
Ten to 12 hours				
Crohn's disease	92 (83·5–99)	3·3 (2·6–3·6)	0·5 (0–2)	7·9 (1–16·5)*
Sarcoid/TB	95 (89·5–98)	3·2 (2·6–3·5)	0 (0–1)	4·5 (1·5–10·5)
Healthy volunteers	94 (85–99)	3·2 (2·9–3·4)	1 (0–5)	4 (1–13)
Twenty-four to 28 hours				
Crohn's disease	99·5 (97–100)	3·3 (2·6–3·8)	0 (0–1)	0·5 (0–6)
Sarcoid/TB	96·5 (92–98·5)	3·2 (2·7–3·5)	0 (0–1)	2 (1–17·5)
Healthy volunteers	97 (91–100)	3·2 (3·0–3·6)	0 (0–2)	1·5 (0–7)

Median value and observed range. Difference compared with healthy volunteers are indicated by *P<0·01 (Mann-Whitney rank sum test for unpaired data.

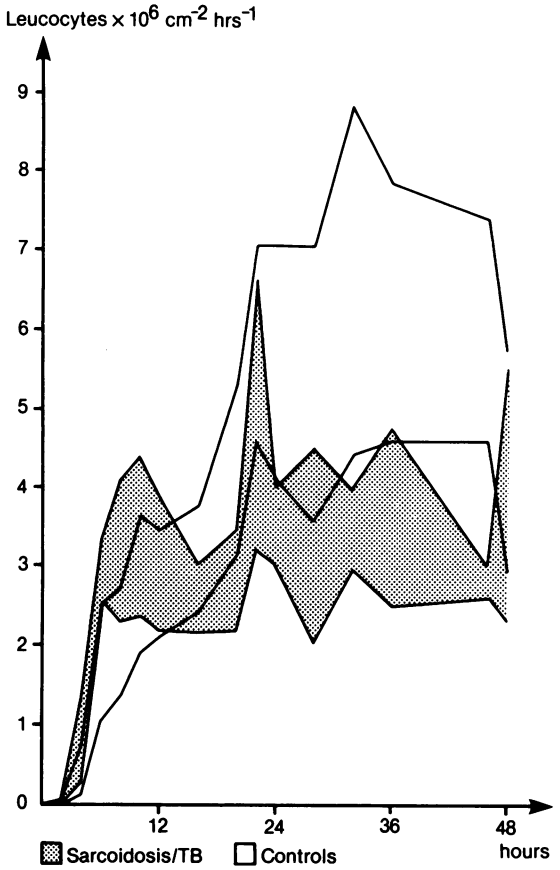

Fig. 3 *Leucocyte migration rate in patients with sarcoidosis/tuberculosis. The area indicated gives the 25/75 percentile.*

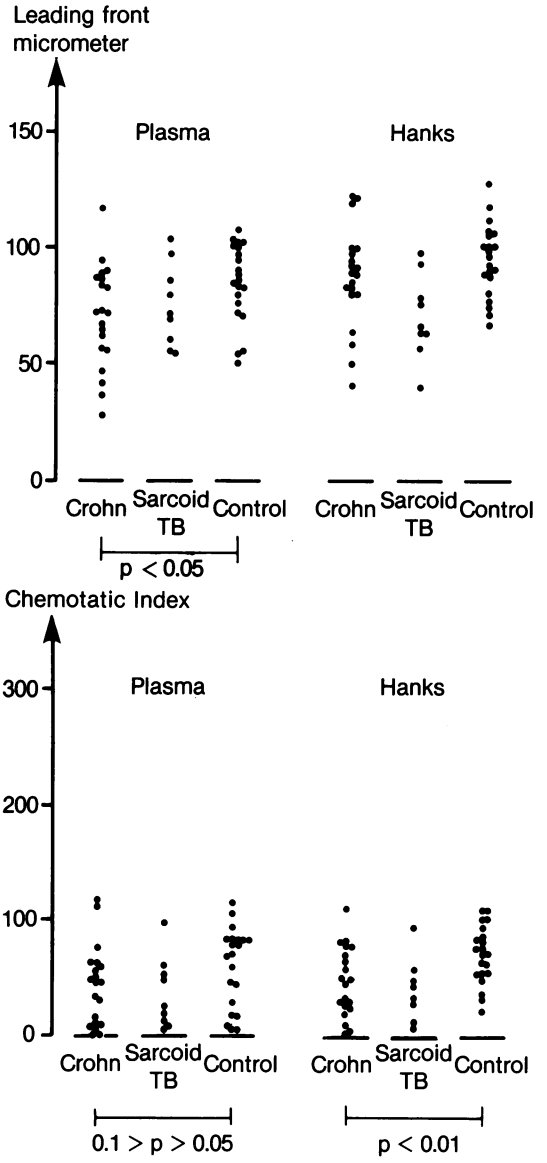

Fig. 4 *Random migration by leucocytes in Boyden chambers in Crohn's disease (N=20), sarcoidosis/ tuberculosis (N=9), and in healthy volunteers (N=21). Values obtained by the leading front method and the migration index are given for leucocytes suspended in autologous plasma and in Hanks's balanced salt solution.*

healthy controls (median 86 μm, $P<0.05$). After washing and resuspension in Hanks's solution, the random migration increased to a median of 90 μm in Crohn's disease (healthy volunteers 97, not significant). The migration index showed no differences between patients with Crohn's disease and healthy volunteers when the leucocytes were suspended in plasma, but if they were suspended in Hanks's solution the migration proved slightly lower in Crohn's disease patients (Fig. 4, $P<0.01$).

Random migration of leucocytes from patients with sarcoidosis/tuberculosis did not exhibit any differences either when measured by the leading front or by the migration index.

Chemotactic response to casein is given in Fig. 5. There was no difference between any of the groups studied. A small increase in the response measured by the leading front was observed after removal of plasma in patients with Crohn's disease (plasma: median 120 μm, Hanks's solution: 130 $P<0.01$). This could not be demonstrated by using the migration index.

Random migration and chemotactic response were not correlated to *in vivo* mobilisation, disease activity, or polymorphonuclear neutrophilic

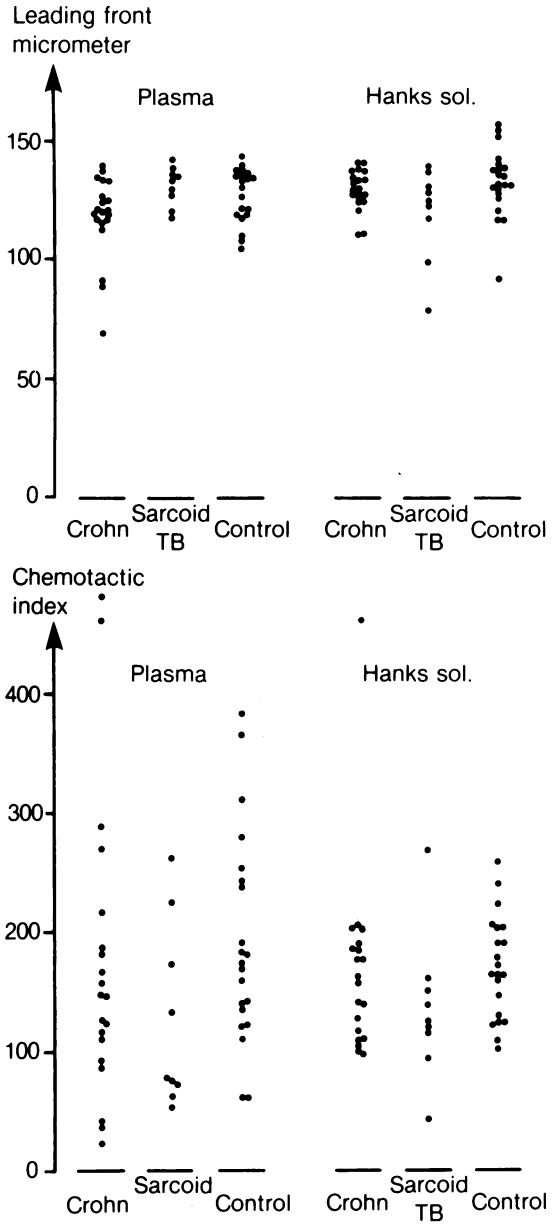

Fig. 5 *Chemotactic response to casein 5 mg/ml by leucocytes from patients with Crohn's disease (20), sarcoidosis/tuberculosis (nine), and from healthy volunteers (21). Values obtained by the leading front method and by the migration index for leucocytes in plasma and in Hanks's balanced salt solution.*

granulocyte count in the patients with Crohn's disease or in the controls.

Phagocytosis

Phagocytosis of bovine serum albumin and of lipopolysaccharide emulsion is summarised in Fig. 6. The phagocytic response showed a wide variation, but no differences between patients with Crohn's disease and the control groups. In patients with Crohn's disease and high clinical activity the leucocytes also showed high phagocytic activity. Serum independent phagocytosis (bovine serum albumin) was positively correlated to the clinical activity index (Spearman rank rho: 0·4812, $P<0·05$). Serum dependent phagocytosis (lipopolysaccharide emulsion) also showed the highest values at a high clinical activity, but did not reach the significance level (Spearman rank rho: 0·4500; $0·05\ P<0·1$).

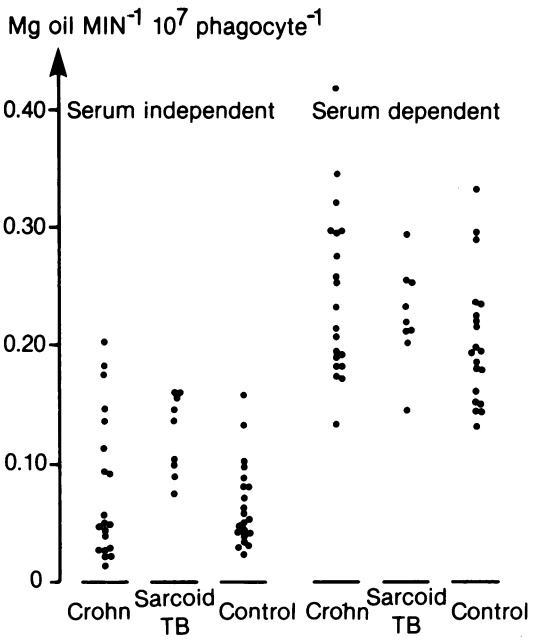

Fig. 6 *Phagocytosis by leucocytes from patients with Crohn's disease (20), sarcoidosis/tuberculosis (nine), and from healthy volunteers (21). Serum independent (bovine serum albumin emulsion) and serum dependent phagocytosis (lipopolysaccharide emulsion) is given.*

Nitro blue tetrazolium reduction

Resting leucocytes generated formazan from nitro blue tetrazolium at higher rates in Crohn's disease and in sarcoidosis/tuberculosis than in healthy volunteers (Fig. 7). Resting leucocyte tetrazolium reduction did not correlate with disease activity, chemotactic or phagocytic activity of the leucocytes.

During phagocytosis patients with Crohn's disease reduced less tetrazolium than did healthy volunteers (Fig. 7). Patients with Crohn's disease showed a median of 0·2928 extinction/mg oil phagocytosed and healthy volunteers 0·4443

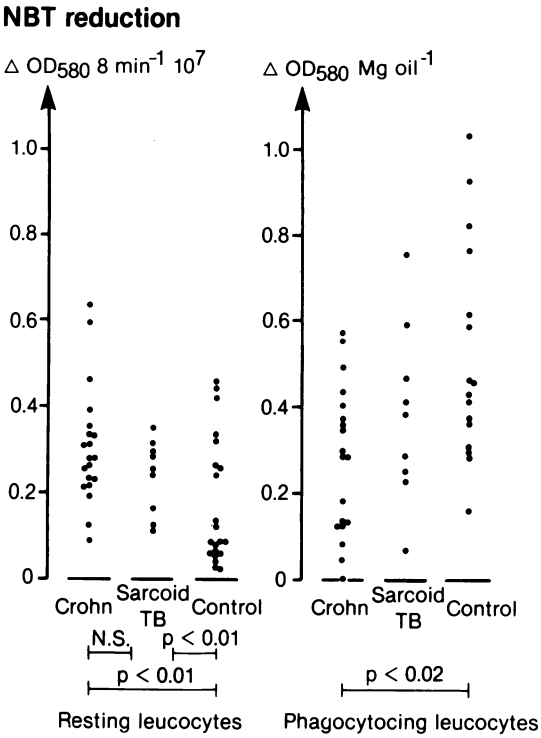

Fig. 7 *Nitro blue tetrazolium (NBT) reduction by leucocytes from patients with Crohn's disease, sarcoidosis/tuberculosis, and from healthy volunteers. Values for resting leucocytes (extinction/8 min/10^7 phagocytes) and phagocytosing leucocytes (extinction/mg oil phagocytosed) are given.*

extinction/mg oil ($P<0.02$). Patients with sarcoidosis/tuberculosis showed values in between healthy volunteers and patients with Crohn's disease.

Discussion

Accumulation of leucocytes is a hallmark of inflammation. In Crohn's disease leucocytes appeared slowly and in reduced numbers in the skin windows, differing in this respect from healthy volunteers as well as from patients with sarcoidosis/tuberculosis. The reduced leucocyte mobilisation was an almost obligatory finding in Crohn's disease and was not correlated with disease activity, duration, or localisation of the disease. Our findings support and extend previous reports[4] and suggest that the reduced leucocyte mobilisation plays an important role in the pathogenesis of the disease.

Leucocyte mobilisation is the result of humoral and cellular factors and depicts the mobilisation to an inflammatory lesion from the blood.[8]

In vitro migration in Boyden chambers did not reveal any changes in the chemotactic migration of leucocytes in patients with Crohn's disease whereas the random migration was reduced. However, this reduction was slight and does not afford a likely explanation for the substantial and constantly reduced leucocyte mobilisation. The inhibition of the *in vitro* migration by plasma in Crohn's disease could hypothetically be caused by activated components of the complement system—predominantly C3 and the properdin system—known to be present often in the plasma of patients with Crohn's disease.[9] Activated components of the complement system—produced by zymosan activation—are known to induce also deactivation of leucocyte migration *in vitro*.[10 11] The chemotactic index did not show a similar influence by plasma, but this is consistent with the leading front method being more sensitive to the effect of fragment of the complement system.[12]

The histological resemblance of the Crohn's disease lesion to the lesion found in patients with chronic granulomatous disease has been noted and is suggestive.[2] Polymorphonuclear neutrophilic granulocytes from patients with chronic granulomatous disease are unable to reduce nitro blue tetrazolium. The polymorphonuclear neutrophilic granulocytes from patients with Crohn's disease reduced tetrazolium at an increased rate in the resting state, but during phagocytosis the reduction was lower per mg phagocytosed material. In other words the higher phagocytosis rate is not associated with a similar augmentation of the reductive potential of the polymorphonuclear neutrophilic granulocytes. Increased spontaneous leucocyte reduction of tetrazolium had previously been demonstrated in Crohn's disease by a qualitative slide technique,[13] and it is present also in other inflammatory states. The lower nitro blue tetrazolium reduction during phagocytosis has not been observed previously. It seems at variance with previous findings,[4] but may reflect the different methods used, especially the correction for differences in phagocytosis done by us. Whether the lower tetrazolium reduction reflects the increased phagocytosis or is due to a defect of the leucocytes is not clear, but the increased resting leucocyte tetrazolium reduction indicates the former explanation.

Phagocytosis by polymorphonuclear neutrophilic granulocytes from patients with Crohn's disease was positively correlated with the clinical activity index. Increased phagocytosis also occurs after unspecific trauma[14] (submitted for publication) and bacterial infections.[15] Therefore, it most probably reflects secondary changes. Phagocytosis

has previously been reported to be reduced in Crohn's disease,[16] but methods used did not allow quantification of the phagocytosis.

The explanation of the reduced mobilisation to skin windows in Crohn's disease, then, is not cellular dysfunction. Inhibitors of leucocyte migration produced in the tissue could contribute to the reaction, but preliminary studies have failed to demonstrate increased lymphokine production by leucocytes from patients with Crohn's disease (K Bentzen, personal communication). A delayed or insufficient release of inflammatory mediators remains to be investigated. The prolonged lag phase observed in the present study favours such a possibility.

The unquestionably poor inflammatory response in Crohn's disease supports the hypothesis that a pathogenetic factor may be antigenic material which—while normally eliminated—gains access to the intestinal wall where it elicits a chronic inflammatory response as a foreign body reaction.

It is a pleasure to acknowledge the assistance of AL Poulsen, H Kargaard, and H Furhauge. Dr T Fløystrup, Head of the Chest Department, Lyngby Hospital, kindly allowed us to study patients under his care. The study was supported by the Danish Medical Research Council (grant No. 512-7257) and by Johan and Hanne Weimann née Seedorf's Foundation.

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